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RNAi-induced targeted silencing of developmental control genes during chicken embryogenesis

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Abstract

The RNA interference technique is a powerful tool to understand gene function. Intriguingly, RNA interference cannot only be used for cells in vitro, but also in living organisms. Here, we have adapted the method for use in the chick embryo. However, this technique is limited by the uncertainty in predicting the RNAi transfection efficiency and site in the embryo. Hence, we elaborated a modified vector system, pEGFP–shRNA, which can coexpress enhanced green fluorescent protein (EGFP) and short hairpin RNA (shRNA) simultaneously to facilitate analysis of gene silencing in chicken embryos. We tested the silencing of two highly conserved genes (*cAxin2*, *cParaxis*), which play crucial roles in chicken embryonic developmental processes. For each target gene, four to five small DNA inserts, each of them encoding one shRNA, were selected and cloned individually to the vector downstream of the Pol III promoter (either human H1 or U6 promoter), which shared with highly conserved motifs in human and chicken. The pEGFP–shRNA constructs were electroporated into the neural tube or somites. After subsequent re-incubation of 24 h, the EGFP expression, with green fluorescent signal, indicated the transfected regions in the neural tube or somites. The EGFP expressing embryos were further submitted into the process of in situ hybridization for examination of the silencing effects. The results show that the EGFP signal in transfected areas correlated with the silencing of the target genes (*cAxin2*, *cParaxis*). The *cAxin2* expression was inhibited by shRNAs of either targeting the RGS domain or the DAX domain coding region. The *cParaxis* mRNA level in transgenic somites and the related migratory myogenic population was also reduced. The results suggest that our novel dual expression EGFP–shRNA system opens a new possibility to study gene function in a convenient and efficient way.

Keywords: RNAi; shRNA and EGFP coexpression; *cAxin2*; *cParaxis*; Neural tube; Somites; Embryos

Introduction

During embryonic development, a tight control of activation and inactivation of the newly formed genome is the prerequisite of cell proliferation, growth, differentiation, pattern formation, and morphogenesis. This becomes manifest by dynamic gene expression patterns, which specify where and when particular genes are expressed in the embryo. In vertebrates, certain genes are expressed during embryonic development in a time- and tissue-specific manner. Axin2 is an RGS and DAX (or DIX) domain containing protein which is expressed in Hensen's node, primitive streak, tail bud, neural tube, dermomyotome, dorso-medial lips of somites, limb buds, AER, and brain. As a DAX domain containing protein, Axin activates the mitogen-activated protein kinase JNK via distinct mechanisms (Wong et al., 2004). As an RGS domain containing and nuclear–cytoplasmic shuttling protein, *Axin* mediates the degradation of *beta-catenin*, a key effector of the canonical *Wnt* signaling pathway, which regulates many aspects of embryonic development (Cong and Varmus, 2004). *Paraxis* is a basic helix–loop–helix transcription factor which is expressed in the paraxial mesoderm and somites. *Paraxis* regulates morphogenesis of the somites that have a central role in the patterning of the axial skeleton and skeletal muscles (Burgess et al., 1995; Sosic et al., 1997). In mice homozygous for a *Paraxis* null mutation, cells from

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the paraxial mesoderm are unable to form epithelia and somite formation is disrupted as a consequence (Burgess et al., 1996).

Regulating or reprogramming of gene expression patterns has a crucial role in embryonic development (Reik et al., 2001). The recent discoveries of RNA interference and related RNA silencing pathways have revolutionized our understanding of gene regulation. The dsRNA-processing enzyme Dicer was found to produce short interfering RNAs (siRNAs), which induce sequence-specific gene silencing. The natural short hairpin dsRNAs (termed as microRNAs or miRNAs) have been described, which link the RNAi machinery to a natural developmental gene regulatory mechanism, involved in developmental timing, cell death, cell proliferation, and cell fate (Ambros, 2004). In fact, the *lin-4* and *let-7*, microRNA genes, were first cloned on the basis of their mutant phenotypes, and the genes were found to encode (21–22 nucleotides) RNAs that are non-coding for any proteins (Lee et al., 1993; Reinhart et al., 2000). A defect of microRNA genes in mice accounted for the developmental phenotypes associated with disruption of the RNAi pathway (Bernstein et al., 2003). Now abundant non-coding RNAs were found from *C. elegans* to humans (Lim et al., 2003; Lewis et al., 2003; Bartel, 2004). In the draft of the chicken genome, 121 microRNAs were predicted and reported (Hillier et al., 2004).

RNAi is a remarkable process whereby small non-coding RNAs silence specific genes. The discovery of the endogenous microRNAs in the form of small temporal RNAs as triggers of the RNAi pathway suggested that exogenous RNA triggers might induce RNAi in animal cells too. The vector-based shRNAs with hairpins ranging from 19 to 29 nucleotide base pairs in length, and with the degrees of structural similarity to natural microRNAs, can be designed based on target gene sequences (Brummelkamp et al., 2002; Khvorova et al., 2003). Synthetic siRNAs, which are similar to the products of Dicer, were shown to induce sequence-specific gene silencing in human cells without initiating non-specific gene silencing (Elbashir et al., 2001). More recently, the RNAi machinery was linked to silencing of chromatin regulation (Volpe et al., 2002; Lee et al., 2004). The success in using RNAi for analyzing single genes has led to efforts to apply this approach on a large scale for functional genomics. A custom microarray platform for analysis of microRNA gene expression is initiated and available. The microRNA expression patterns from different embryonic stages, embryonic stem cells, and embryoid bodies were examined (Thomson et al., 2004). Recently, also, methods have been described for constructing shRNA libraries based on manipulation of complementary DNA or genomic DNA (Paddison et al., 2004; Sen et al., 2004). However, some gene functions, particularly those related to development and disease may be difficult to be identified without experiments in vivo.

After the genomes of several species were assembled, the gene sequences of interest are available. RNAi, harnessed as an experimental tool, has revolutionized approaches to decode gene function (Hannon and Rossi, 2004). Vector-based shRNAs have been tested to interfere with specific target genes in chicken embryos (Pekarik et al., 2003; Katahira and Nakamura, 2003; Chesnutt and Lee, 2004). With the development of vector-based shRNA as a tool for specific gene silencing, the chicken embryo may become an efficient in vivo model system to study gene function during embryonic development. This approach will be one of the valuable tools for functional genomics. However, before shRNA could be harnessed as an efficient experimental tool for silencing specific genes in chicken embryos in vivo, we are facing considerable questions. First, we want to know whether the human U6 promoter or H1 promoter can drive shRNA synthesis in chicken embryos, although it has been reported that this is the case for the mouse U6 promoter (Katahira and Nakamura, 2003). Secondly, when trying to apply exogenous shRNA to trigger silencing of target genes in vivo, it is difficult to identify transgenic cells or tissue in embryos without the coexpression of a reporter gene. In order to meet these challenges, we developed a human Pol III promoter driven shRNA and SV40 promoter driven EGFP coexpression system, and tested the constructs in the chicken embryo model to generate a somatic loss-of-function approach for the chicken *Axin2* (*cAxin2*) and chicken *Paraxis* (*cParaxis*) genes.

Materials and methods

Analysis of target genes for shRNA selection

To find shRNA target sites in the mRNAs of *cAxin2* (NM_204491) and *cParaxis* (NM_204946) genes, the coding sequences of genes were analyzed online with pSilencer™ Expression Vectors Insert Design Tool, which were provided by Ambion company (www.ambion.com). The program scanned the sequences, and indicated each position of the AA dinucleotides, and the following 19 base target oligonucleotides. The candidate shRNAs were subjected for BLAST analysis on Genbank from NCBI server (www.ncbi.nlm.nih.gov/BLAST). Four to five candidate siRNAs for each target gene were selected. As shRNA DNA template and plasmid insert, the loop sequence (TTCAAGAGA) between the corresponding sense and antisense siRNA oligonucleotides; poly T, and overhangs for cloning were added as outlined in the pSilencer siRNA expression vectors' Instruction Manuals from Ambion. The designed DNA oligonucleotides were ordered from Invitrogen.

Construction of pEGFP–shRNA plasmid

The p*Silencer* 3.0-H1 and the 2.0-U6 siRNA expression vectors were provided by Ambion. Each p*Silencer* Vector has an RNA polymerase III (Pol III) promoter, ampicillin resistance gene, and *E. coli* origin of replication. Both

vectors were linearized with *Bam*HI and *Hin*dIII, which leave overhangs that facilitate directional cloning; and 1× DNA Annealing solution was prepared for annealing the DNA oligonucleotides as an insert for ligation.

The selected siRNA inserts for the gene silencing were subcloned into the pSilencer3.0-H1 vector. For comparing the efficacy of H1 promoter and U6 promoter to drive shRNA synthesis in chicken embryos, the same selected siRNA inserts for *cAxin2* gene silencing were not only subcloned into the p*Silencer* 3.0-H1, but also subcloned into the p*Silencer* 2.0-U6, as these two kinds of vectors shared the same overhangs as the siRNA inserts at the 5′ and 3′ end of the DNA with *Bam*HI and *Hin*dIII restriction sites. After ligation, the plasmids with inserts were confirmed by sequencing (MWG Biotech) with the primer (5′ CGGGCCTCTTCGCTATTACG 3′), which is shared by these two vectors. The circular, negative control p*Silencer* vector that expresses a siRNA with limited homology to any known sequences was designed and provided by Ambion.

To further construct the pEGFP and shRNA coexpression system, we took the fragments from shRNA insert containing pSilencer 3.0-H1 or 2.0-U6 plasmids by restriction with *Kas*I and *Alw*NI. The fragments contained H1 or U6 promoter, shRNA sense, loop, antisense, and 6 T termination sequence as RNA pol III terminator. These fragments were then reorganized into a modified green fluorescent protein expression vector with extra *Kas*I restriction site at the end of *E. coli* origin of replication. The vector was modified on the base of pCMS-EGFP vector (BD Biosciences), which contains SV40 promoter, enhanced green fluorescent protein gene (EGFP), ampicillin resistance gene, and *E. coli* origin of replication.

In ovo electroporation

Fertilized eggs of *Gallus gallus* (White Leghorn) were incubated at 38°C and 80% relative humidity for the required time period to obtain stages 17–18 HH. The stages of embryos were determined according to Hamburger and Hamilton (1951). The upper side of the eggs were windowed to visualize the embryos, the extra membrane were partially removed. The pEGFP–shRNA constructs (2–3 μg/μl) are dissolved in an adjunct solution (50 μl stock solution: 25 μl carboxymethlycellulose, 7.5 μl fast green, 3.0 μl $MgCl_2$, 7.5 μl 20× PBS, and 7.0 μl H_2O) in a ratio of 2:1. The DNA constructs were microinjected into the target neural tube or somites, and electroporated as previously described (Mann et al., 2003; Scherr et al., 2003). The electrodes were placed at each side of the microinjected embryo, and five square pulses of 30–55 V, 20-ms width was applied for each embryo. Upon passing current, the plasmid DNAs with negative charges were sent to the cells or tissues adjacent to the anode side. After 24-h re-incubation, the EGFP expression in the transgenic embryos was visualized under fluorescence microscopy and photographed.

In situ hybridization

The EGFP expressing embryos were fixed overnight in 4% paraformaldehyde at 4°C, and then passed through graded series of methanol dehydration and stored at −20°C. Whole mount in situ hybridization with a probe against the target gene was performed as previously described (Nieto et al., 1996). As a template for riboprobe preparation to detect *cAxin2* gene expression, the *cAxin2* coding sequence was used. Probe for cParaxis was prepared from *cParaxis* gene coding region. All of the probes were labeled with digoxigenin RNA labeling kit (Roche). Alkaline phosphatase conjugated anti-DIG (Roche), with the color substrates 4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP), was used for detection of target gene expression signals. The chicken embryos displaying a restricted expression pattern of the target gene were photographed again. The images from the whole mount in situ hybridization were analyzed, comparing with the EGFP signals from the same transgenic embryos.

Results

Homologous sequences of U6 and H1 promoters

High rates of pol III transcription of RNA genes are necessary for cells to sustain growth. The well-characterized promoters of RNA genes transcribed by RNA polymerase III are from the human H1 and U6 RNAs (Myslinski et al., 2001; Stunkel et al., 1997). Both U6 and H1 RNA genes are involved in regulation of post-transcription processes. The U6 RNA links to the function of spliceosomes, which catalyze removal of the non-coding intron from a pre-mRNA. H1 RNA is the RNA component of human Ribonuclease P (RNase P), which cleaves the 5′ end of precursor tRNA (Mann et al., 2003). The DNA elements of the human H1 or U6 RNA promoters both contain the distal sequence element (DSE), proximal sequence element (PSE), and TATA motifs (Myslinski et al., 2001; Stunkel et al., 1997). The PSE together with the TATA box determines the assembly of a pol III-specific pre-initiation complex for RNA gene transcription (Cabart and Murphy, 2001). After the draft genomes of human, chimpanzee, and mouse were assembled, the draft genome sequence of the red jungle fowl, *G. gallus*, was presented recently in *Nature*. Within the draft chicken genome, a total of 15 copies of U6 RNA genes and 1 copy of RNase P RNA gene were identified (Hillier et al., 2004). The comparison of chicken sequences with the sequences of human and the other species may provide high specificity in predicting functional elements. Here, we compared the homologous sequences of human U6 and H1 promoters from human, chimpanzee, mouse, rat, and chicken. All of the U6 RNA promoters contain the DSE, PSE, and TATA motifs. All the species share the sequences of TTTGCA in the DSE motif, CC in the PSE motif, and a TATA box in TATA motif.

From all compared species, the H1 RNA or H1 homologous promoter sequences also contain the DSE, PSE and TATA box motifs. However, they are organized in a more compact way (the 5′-flanking region between around −100 and −1) than in U6 promoters (the 5′-flanking region between around −200 and −1). Furthermore, the sequences in the DSE motif of H1 or H1-like promoters are relatively similar. Human and chimpanzee share the sequence of ATTTGCA, chicken has one point mutation A in its DSE motif resulting in the sequence ATTAGCA. However, mouse and rat have only four identical residues TGCA in the forward orientation sequence (ATGCAAA) of their DSE motifs (or the identical residues if their DSE motif sequences are in the reverse orientation) to human, chimpanzee, and chicken. So, compared with human U6 and H1 promoters, chicken has the DNA elements for Pol III to transcript RNA genes in its homologous sequences (Fig. 1).

Vector-based shRNA and EGFP coexpression system

With developmental application of RNAi techniques on chicken embryos in vivo, we tried to build up a vector-

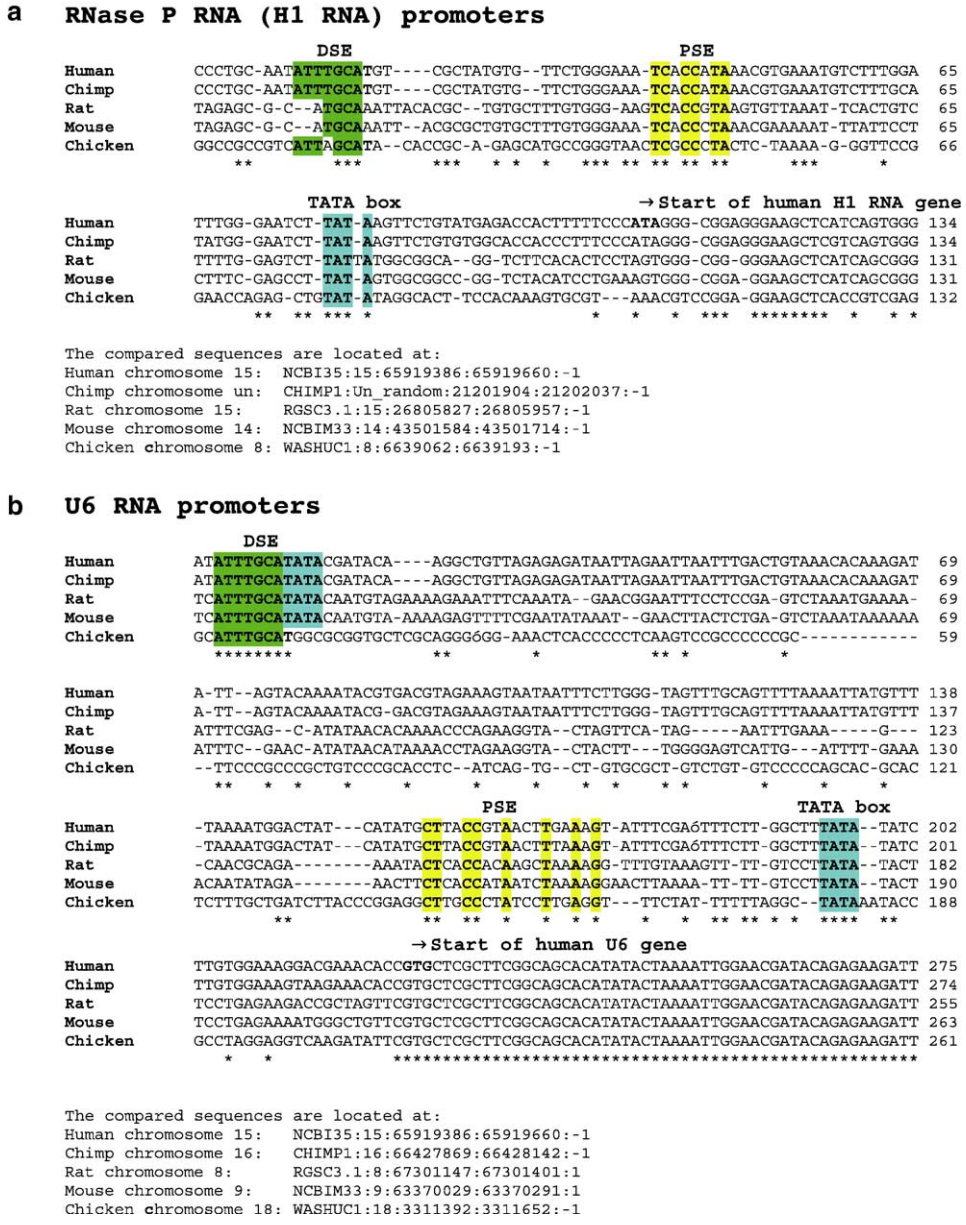

Fig. 1. Motif analysis of human RNase P (H1) RNA or U6 RNA promoter homologous sequences from the assembled human, chimpanzee, mouse, rat, and chicken genomes (all of the sequences located in genomes were indicated). Both RNase P (H1) and U6 promoters contain DSE, PSE, and TATA motifs in all of the compared species. (a) Alignment of RNase P (H1) RNA gene promoters: In DSE motifs, human and chimpanzee have the sequence of TTTGCA; chicken has one point mutation as the sequence of TTAGCA; rat and mouse have the reverse sequences, compared with consensus sequence of DSE motif. The start codon of human H1 RNA gene was **ATA**. (b) Alignment of U6 RNA gene promoters: All the species have highly conserved DSE motifs with the sequence of TTTGCA. The start codon of human U6 RNA gene was **GTG**.

based EGFP and shRNA coexpression system. To this propose, we took two genes related to development, *cAxin2* (NM_204491) and *cParaxis* (NM_204946), which we are familiar with, for these experiments. These two genes have different molecular characteristics and gene functions, as well as specific gene expression patterns in chicken embryos.

To find efficient shRNAs for silencing of target genes of interest, the coding sequences of *cAxin2* (2514 bp) and *cParaxis* (552 bp), from the beginning with the AUG start codon of the transcript to the stop codon, were scanned with pSilencer™ Expression Vectors Insert Design Tool from Ambion. For gene silencing, double AA plus the 3′ adjacent 19 nucleotides represented one of the potential shRNA target binding sites. siRNAs with 3′ overhanging UU dinucleotides were reported to be the most effective (Elbashir et al., 2001). We have analyzed the candidate shRNAs with BLAST based on Genbank to know the contiguous base pairs of homology to other genes. We have noticed that the suggestion on shRNAs sequence conditions for effective gene silencing in mammalian cells and in ovo RNAi using chicken embryos. It included the G/C at the 5′ end of the sense strand; A/U at the 5′ end of the antisense strand; at least five A/U residues in the 5′ terminal of the

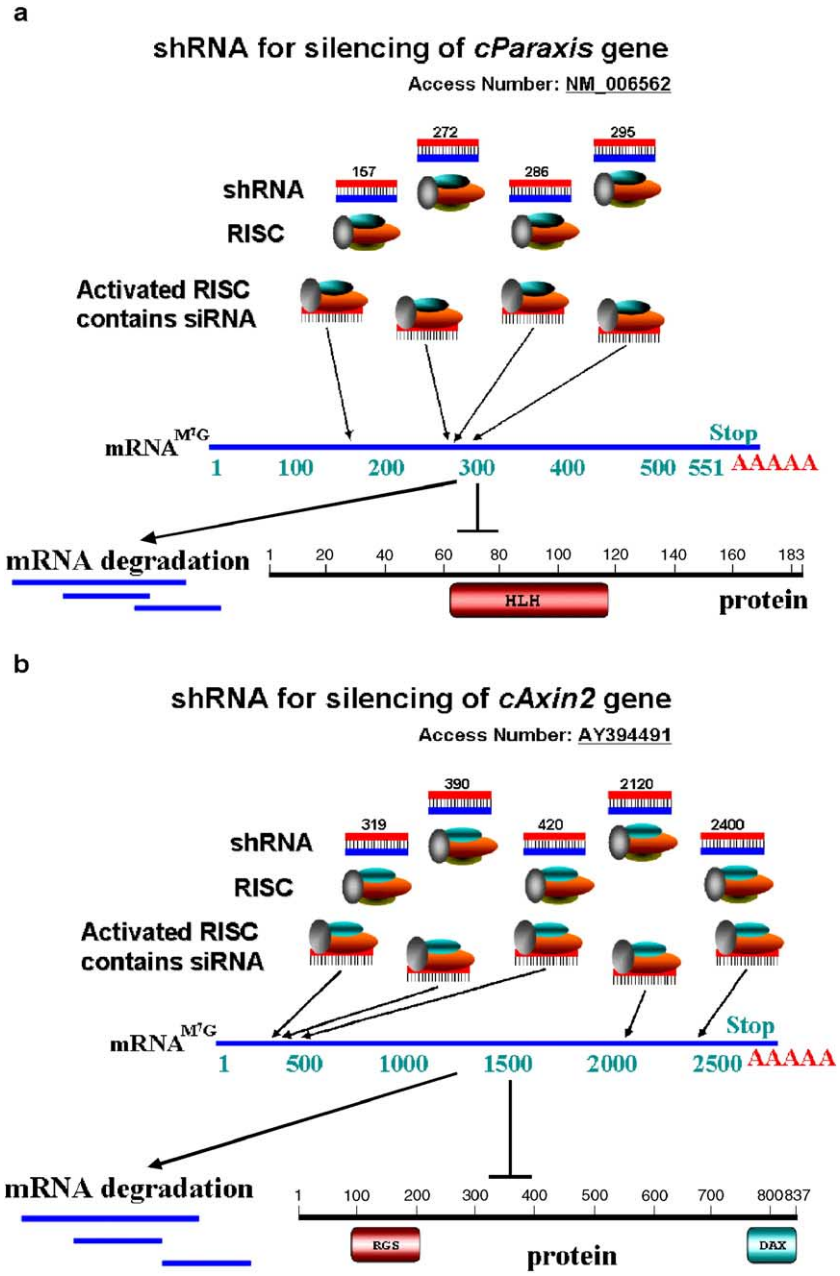

Fig. 2. Analysis of target genes (*cParaxis* and *cAxin2*) and selected shRNAs for silencing the target genes are indicated by diagrams. (a) Selected shRNAs are against 5′ end (157) and bHLH domain coding region (272, 286, 295) of the *cParaxis* mRNA. (b) Selected shRNAs are against the RGS domain coding region (319, 390, and 420), or the DAX domain coding region (2120, 2400) of *cAxin2* mRNA.

antisense strand; and the absence of any GC stretch of more than 9 nt in length (Ui-Tei et al., 2004). Therefore, to interfere with *cParaxis*, we selected four DNA oligonucleotides as shRNA templates (the oligonucleotides of the sense strands begin at positions 157, 272, 286, and 295 from the start codon AUG) (Fig. 2a). To interfere with the *cAxin2* gene, we selected three DNA oligonucleotides from the *cAxin2* RGS domain coding region (the oligonucleotides of the sense strands begin at positions 319, 390, and 420 nt from the start codon AUG), and two oligonucleotides from *cAxin2* DAX domain coding region (the oligonucleotides of the sense strands begin at positions 2120 and 2400 nt from the start codon AUG) (Fig. 2b). The potential target sites were compared to the gene database and the homologies of selected shRNAs for *cAxin2* were fewer than 16 nt contiguous base pairs to the other genes, even to chicken *Axin1* (AY687628 and AY640375).

The selected DNA inserts were first ligated into the pSilencer 3.0-H1 or pSilencer 2.0-U6 vectors and sequenced with expected results. However, when we applied those vector-based shRNA to chicken embryos, we found that it was difficult to identify the transgenic cells or tissue after the plasmid delivering and re-incubation of those embryos. This led us to design the vector-based EGFP and shRNA coexpression system to overcome this obstacle. As EGFP expression vector, pCMS-EGFP shared the sequence of ColE1 origin with the vectors pSilencer 3.0-H1 or pSilencer 2.0-U6. This gave us a chance to harvest the fragments from the insert containing pSilencer 3.0-H1 or pSilencer 2.0-U6 plasmid by double restriction with enzymes *Alw*NI and *Kas*I. The one kind of harvested fragments from constructed pSilencer 3.0-H1 plasmids contained the elements of *Kas*I restriction site, H1 promoter, sense, loop, antisense, poly (T), partial 3′ ColE1 origin, and *Alw*NI restriction site sequences. The other kind of fragments from constructed pSilencer 2.0-U6 plasmids contained the same elements, but with U6 promoter in the opposite orientation (Fig. 3). The fragments were inserted into a modified EGFP expression vector. The plasmids with both shRNA and EGFP coexpression elements have three *Hin*dIII restriction sites, and can be digested into three DNA fragments (415 bp, 1621 bp, and 3884 bp) after restriction with enzyme *Hin*dIII.

EGFP expression indicates the transgenic cells and tissue of the embryos

In ovo electroporation to deliver the plasmid DNA into chicken embryos was reported as an efficient gene trans-

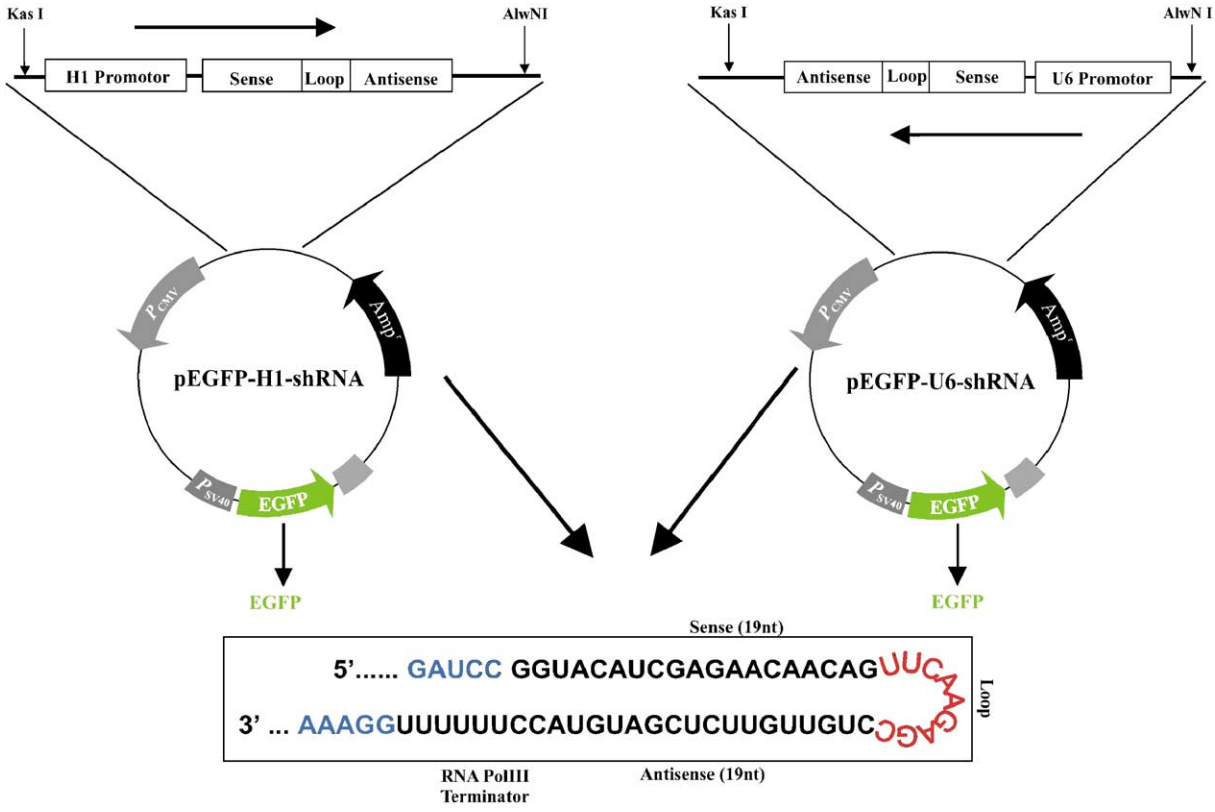

Fig. 3. Plasmid backbones were designed for construction of pEGFP-U6-shRNA and pEGFP-H1-shRNA plasmids. The diagram shows the design and sequence of a sample template (shRNA 420 for silencing *cAxin2* gene). Both plasmids contain EGFP gene expression elements: SV40 promoter, EGFP gene, and poly A. Both plasmids have the insert as DNA template elements for shRNA: sense, loop, antisense, and poly T as RNA pol III terminator (top backbones), but with different Pol III promoters: human H1 RNA promoter (left plasmid diagram) or human U6 RNA promoter with the other orientation (right plasmid diagram). Both kinds of the plasmids are designed to synthesis of same shRNA with 19 base pair (bottom box).

fection method (Pekarik et al., 2001; Katahira and Nakamura, 2003; Chesnutt and Lee, 2004; Krull, 2004; Scaal et al., 2004). We have used this method to send an EGFP expression plasmid, pCMS-EGFP, at a final concentration of 1–2 μg/μl to the neural tube or somites, respectively. Upon passing the current via electroporation, the plasmid DNAs with negative charges were mainly moving into the cells or tissues adjacent to the anode. After the operations and 24-h re-incubation, the embryos were checked under fluorescence microscopy. As expected, the EGFP was expressed both in the sites of transgenic neural tube and somites (Fig. 4). For RNAi of the target genes, *cAxin2* and *cParaxis*, we used our constructed EGFP–shRNA coexpression plasmids at the final concentration of 1–2 μg/μl for gene transfection into the chicken embryos. As *cAxin2* gene is mainly expressed in the neural tube and *cParaxis* gene is strongly expressed in the somites at stages 17–18 HH of chicken embryos, we aimed our constructed plasmids, pEGFP-*cAxin2*-shRNAs, at the neural tubes, and the plasmids, pEGFP-*cParaxis*-shRNAs at the somites.

Target gene silencing in the indicated region with EGFP expression

As the *cAxin2* gene is more than 2.5 kb long, we tested the *cAxin2* gene silencing with our constructed pEGFP-*cAxin2*-shRNA plasmids to target in the area of the RGS coding region or DAX domain of the *cAxin2* gene, separately. After electroporation of the plasmids into the neural tubes and 24 h of re-incubation, the green fluorescent signal from EGFP expression of all plasmids indicated the transgenic sites in the neural tubes. The whole mount in situ hybridization, using *cAxin2* gene-specific antisense probe, was used to check whether *cAxin2* expression was inhibited or not. We found that the *cAxin2* gene expression only in the transgenic sites of neural tubes was inhibited. The inhibition effect was achieved either by targeting on the RGS domain coding region (at the positions of 319, 390, 420), or on the DAX domain coding region (at positions of 2120, 2400) of *cAxin2*. Moreover, the silencing effects of *cAxin2* were achieved by shRNAs driven by either H1 promoter or U6 promoter. As wild-type *cParaxis* is mainly expressed in the somites, we electroporated the four mixed pEGFP-*cParaxis*-shRNAs into somites. After 24 h of re-incubation, we observed a decreased gene expression level of *cParaxis* in transgenic somites and the migratory myogenic population (Fig. 5). Comparison of the images of EGFP expression and silencing of *cAxin2* or *cParaxis* in the transgenic tissues showed that EGFP expression could indicate perfectly well the transgenic cells and tissue with the target gene RNAi in the chicken embryos. Additionally, we also analyzed for specificity of our silencing constructs by electroporating shRNA targeted against *cAxin2* in the neural tubes and checked for *cPax3* and *cCXCR4*, which are also expressed in the neural tube. The expression pattern of both *cPax3* and *cCXCR4* were not affected after interference with shRNA targeting *cAxin2* (data not shown).

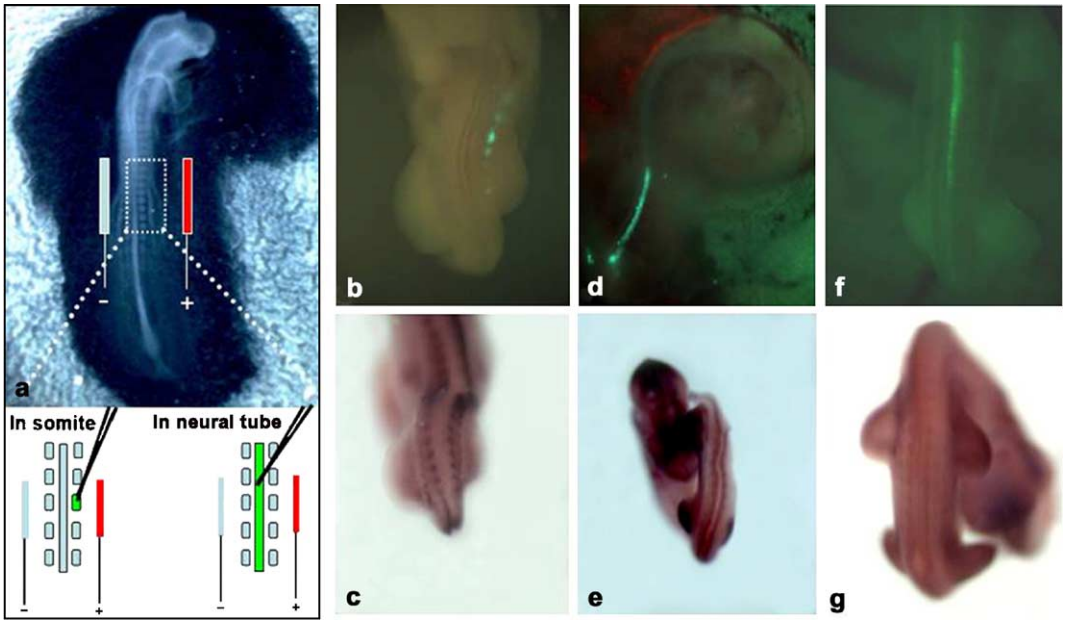

Fig. 4. EGFP gene was expressed in the transgenic neural tubes and somites of chicken embryos after in ovo electroporation of control plasmids (pEGFP-H1 or pEGFP-U6, but without shRNA template). Diagram of in ovo electroporation. The EGFP expressing plasmid can be injected and electroporated into somites or neural tube of chicken embryos as illustrated in panel a. EGFP gene was expressed in somites after transfection with pEGFP-H1 control plasmid in panel b. In the same embryo, *cParaxis* gene was expressed as wild type in somites in panel c. EGFP gene was expressed in neural tubes after the gene transfection either with pEGFP-H1 control plasmid in panel d, or with pEGFP-U6 control plasmid in panel f. The *cAxin2* gene was expressed as wild type in the area of the transgenic neural tubes in panels e and g. Scale bar: in panel b, 0.1 cm for panels b and c; in panel d, 0.1 cm for panels d and e; in panel f, 0.1 cm for panels f and g.

Discussion

Recently, RNAi as an experimental tool has revolutionized approaches to understand gene function. Exogenous dsRNA, siRNA, and shRNA can silence specific target genes. In the cell, long dsRNAs are cleaved into small interfering RNAs (siRNAs), by a ribonuclease Dicer. The siRNAs subsequently assemble with protein components into an RNA-induced silencing complex (RISC), and cause unwinding in the process. Activated RISC then binds to complementary mRNA by base pairing interactions between the siRNA antisense strand and the target mRNA. The bound target mRNA is cleaved and sequence-specific degradation of mRNA results in target gene silencing. In mammalian cells, introduction of long dsRNA (>30 nt) may initiate a potent antiviral response. Although RNAi silences gene expression in a sequence-specific manner, several recent studies have suggested that the specificity of silencing is not absolute (Hannon and Rossi, 2004; Clayton, 2004). Long dsRNA could be cut to produce several tens of small RNAi, some of them may nonspecifically target the other genes which share similar sequences. The efficiency and specificity of RNAi depend on the position and sequence of the shRNA used. The nonspecific response may be reduced by the introduction or expression of siRNAs. Based on the *cParaxis* gene coding sequence, about a dozen of shRNA probes are listed as candidates to target *cParaxis*. They may also be the siRNA products of the long dsRNA in vivo. We tested four shRNA probes (p157, p272, p286, p295) for targeting the *cParaxis* mRNA. The *cParaxis* gene expression was reduced in the transgenic area of the somites, and the related migrating cells in the limb bud. *cParaxis* belongs to the basic helix–loop–helix (bHLH) transcription factor family, in which several genes share a similar sequence in the bHLH coding region. A similar gene is also expressed in somites, *cScleraxis*, the sequence of which is nearly identical to *cParaxis* within the bHLH region but diverges in its amino and carboxyl termini (Burgess et al., 1995, 1996; Sosic et al., 1997). As previously reported by Ambion, typically more than half of randomly designed siRNAs provide at least a 50% reduction in target mRNA levels and approximately 1

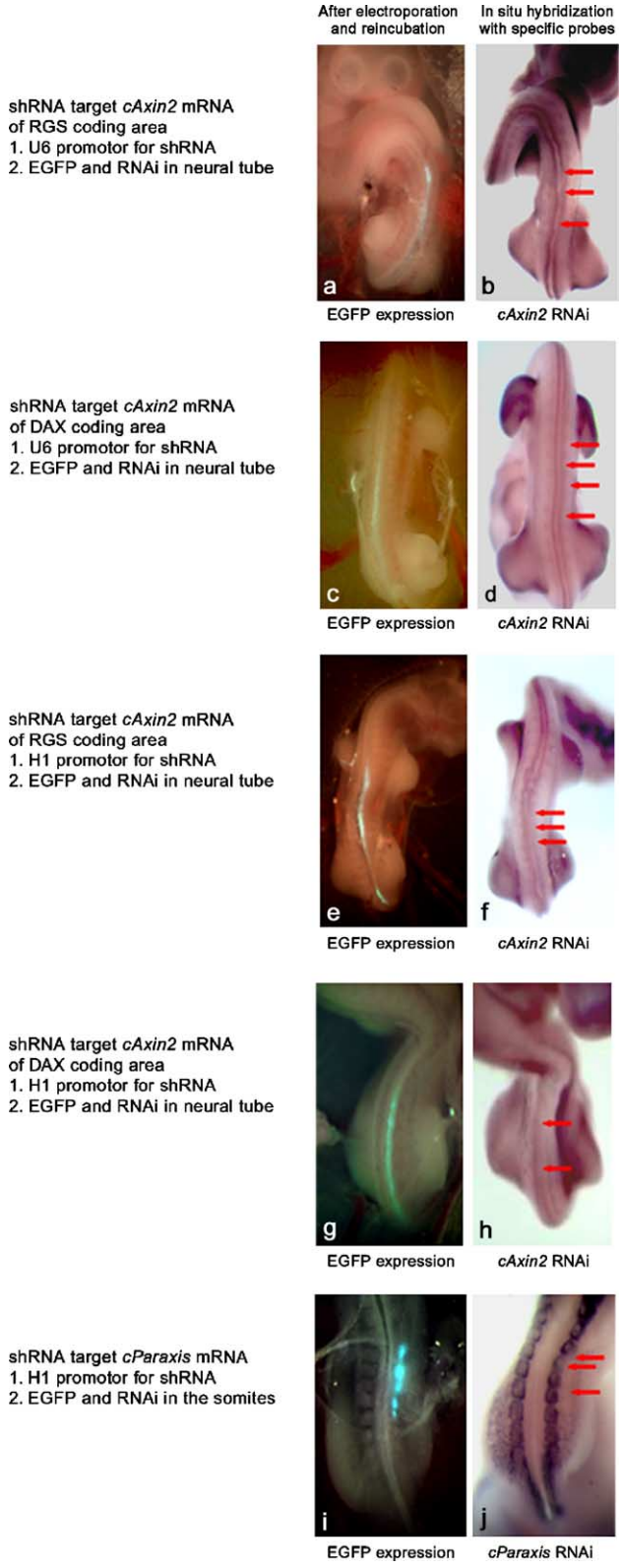

Fig. 5. Comparison of EGFP gene expression and target gene silencing in the same transgenic embryos with coexpressing pEGFP–shRNA plasmids. In the transgenic neural tube with EGFP gene expression in panel a, the *cAxin2* gene expression was interfered, red arrows in panel b, the shRNA synthesis were driven by U6 promoter, and the shRNA binding sites were selected around RGS domain. The EGFP gene was expressed in neural tube in panel c, the *cAxin2* gene expression was inhibited, indicated by red arrows in panel d, the shRNA synthesis was driven also by U6 promoter, but the shRNA binding sites were against the DAX domain. The EGFP gene was expressed in neural tube in panel e, the *cAxin2* gene expression was down-regulated shown by red arrows in panel f, the shRNA synthesis was driven by H1 promoter, the shRNA binding sites were against the RGS domain. The EGFP gene was expressed in neural tube in panel g, the *cAxin2* gene expression was inhibited, red arrows in panel h, the shRNA driven by H1 promoter, but the shRNA binding sites were against the DAX domain. In the transgenic somites with EGFP gene expression in panel i, the *cParaxis* gene expression was interfered, indicated by red arrows in panel j, the shRNA synthesis was driven by H1 promoter, and the shRNA binding sites were against regions lying before and in the bHLH domain. Scale bar: in panel a, 0.1 cm for panels a and b; in panel c, 0.1 cm for panels c and d; in panel e, 0.1 cm for panels e and f; in panel g, 0.1 cm for panels g and h; in panel i, 0.1 cm for panels i and j.

of 4 siRNAs provides a 75–95% reduction. Multiple applications of shRNAs may result in relatively high silencing efficiency. In this study, we tested two or four mixed shRNAs to silence target genes. Based on BLAST analysis of the gene database, we find that the supposed shRNAs for silencing *cParaxis*, p157, p272, p286, and p295, are completely complementary to *cParaxis*. When we eletroporated these shRNA constructs precisely to *cParaxis* expressing cells in somites at particular times and locations, the expression of *cParaxis* could be inhibited. However, the shRNA p286 may interfere with *cScleraxis* (18 nt/19 nt), *cNeuroD* (17 nt/19 nt); and the shRNA p295 may silence *cScleraxis* (18 nt/19 nt), *cNeuroD* (18 nt/19 nt), and *cNeurogenin2* (17 nt/19 nt). As we have known, the *cNeuroD* gene is mainly expressed in the neural tube, *cParaxis* and *cScleraxis* are normally expressed in the somites. The exact location of expression further depends on the stage of development. *cParaxis* expression declines soon after sclerotome formation, whereas *cScleraxis* expression increases in the sclerotome and its derivatives (Burgess et al., 1995). A variety of morphological effects can be expected depending on the time and site of shRNA vector administration. In the in vivo model, we may maximally reduce nonspecific interference of target genes as its expression is time-dependent and tissue-specific during embryonic development.

After the genomes of several species (human, chimpanzee, mouse, rat, and chicken) were assembled, identification of the gene function and gene expression regulation patterns became a major task. From the draft chicken genome data, about one billion DNA base pairs, the chicken genome is only one-third the size of the human genome, but it contains 20,000 to 23,000 genes (Hillier et al., 2004). The genes are expressed in a strictly controlled spatio-temporal pattern during the period of embryonic development. Many of these developmental control genes are also expressed during pathological processes. *cAxin2*, for example, is mainly expressed in the neural tube, and the limb buds, but *cParaxis* is strongly expressed in the somites of embryos. To understand the gene function, in vivo experiments are indispensable. Chicken embryos serve as a good model for research in functional genomics for several reasons: First, it is easy to be handled and accessible to microsurgical manipulation. Secondly, many chicken genes, like *cAxin2* and *cParaxis*, are very similar to human genes. In this study, we have used the human H1 promoter and the human U6 promoter to drive the shRNA for silencing chicken target genes. The results showed that both human H1 and U6 promoter could drive shRNA synthesis in transgenic chicken embryos. Thirdly, compared with the creation of gene knockouts in mice, which can take up to several months, RNAi in chicken embryos can potentially produce answers within days or weeks (Clayton et al., 2004). For some important genes, the generation of knockout mice encounters severe problems due to early embryonic lethality or problems in the construction of homologous recombinants, requiring additional strategies. Alternatively, the techniques of transgenic RNAi were also successfully applied in mouse embryos (Stein et al., 2003; Svoboda et al., 2004).

The chicken embryo has been long used to study gene activity and disorders. After the chicken genome was assembled, the chicken may provide a good model for studying changes in genes linked to diseases like muscular dystrophy, viral infection, aging, and death. Knowledge of chicken gene function will lead to insights into human biology and medicine. RNAi and chicken embryo model may be useful for some related research on human disease. In a remarkably short time since the discovery of RNAi, it has emerged as a powerful tool for the study of gene function. With the increasing use of RNAi in vivo, RNAi has been proposed as a research tool and a potential treatment for human diseases, like cancer, or viral infections. Using RNAi to target genes expressing oncogenic fusion proteins, such as the Bcr-Abl oncoprotein p210 that is characteristic of chronic myelogenous leukemia (CML), has provided excellent proof of principle for RNAi as a therapeutic anti-cancer agent (Scherr et al., 2003). RNAi has been used to target several early and late HIV-encoded RNAs in cell lines and in primary hematopoietic cells (Jacque et al., 2002). Furthermore, hepatitis viruses, like hepatitis B virus (HBV) and hepatitis C virus (HCV), have also been the important targets for potential RNAi therapy. The study reported that significant interference with the HBV and HCV could be achieved by the shRNA (McCaffrey et al., 2003; Kapadia et al., 2003). However, the delivering efficiency of shRNA to infected cells or tissues with disease caused by virus still needs more in vivo research data before clinical studies can begin. On the other hand, chicken embryos can be infected easily by retroviruses, including overexpression of genes of interest by an experimental model of avian retrovirus overexpression system (RCAS and RCASBP) (Hu et al., 2004). The shRNA and chicken embryo model may be a valuable tool to test RNAi to inhibit viral gene expression and viral replication. Additionally, our pEGFP–shRNA coexpression system, using the human H1 promoter or the human U6 promoter, can be optimally used for human cells or human cancer cell lines for screening disease-related genes.

The key challenge for achieving effective RNAi in vivo is its delivery to the desired organ and into the target cells, to ensure specificity and adequate dose. The advantages of using a vector-based EGFP–shRNA coexpression system may make it a more convenient and efficient way to target genes by shRNA-induced silencing in the chicken embryo by in ovo electroporation. Our pEGFP–shRNA vector contains an RNA polymerase III (Pol III) promoter and EGFP expression elements. We use shRNA instead of dsRNA to limit nonspecificity. shRNAs, driven by Pol III promoter (human H1 promoter or human U6 promoter), inhibited the expression of target genes (*cAxin2*, *cParaxis*) in chicken embryos. The EGFP signal indicated the transfected area. It facilitated analysis of gene silencing in the transfected region after electroporation and re-incubation of

the chicken embryos. The effects of RNAi on the expression of target genes were examined by in situ hybridization after the EGFP signals were documented. The result analysis showed that the RNAi effects correlated to cells or tissues with EGFP expression. This novel dual expression EGFP–shRNA system and chicken embryonic model opens new possibilities to study gene function in a convenient and efficient way.

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